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TITLE: Dietary Fish Oil in Reducing Bone Metastasis of Breast Cancer

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14. ABSTRACT

To study the mechanism of inhibitory effect of fish oil on growth of breast cancer cells last year wereported that fish oil or w-3 polyunsaturated fatty acids (PUFAs) (1) increase the level of tumor suppressor proteinPTEN, (2) inhibit the activity of PI 3 kinase, thus blocking a potent growth promoting signaling pathway and (3)increase gene expression of BMP-2. In our final report we show that fish oil diet had significantly increased thesignals leading to breast cancer cell apoptosis. The tumors arising from the fish oil diet fed animals have decreasedexpression of antiapoptotic Bcl-2 and BclXL, increased expression of cytochrome-c and activation of caspase 3,indicating increased apoptosis. Results from our in vivo pilot study, using a nude mouse heart injection model,suggest that fish oil diet can also slow down bone metastasis of the breast cancer cells. Based on our previous reportwe performed a pilot in vivo experiment to study the role of BMP-2 on bone metastasis. The data suggest that BMP-2 can inhibit bone metastasis. The in vivo data however needs to be verified using larger animal pool and statisticalanalysis.

15. SUBJECT TERMS
Omega 3-fatty acids; Bone morphogenetic protein-2 (BMP-2); breast cancer bone metastasis

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Introduction:

This year to further elucidate the mechanism of fish oil mediated reduction in breast tumor growth, we focused on the activation of tumor cell apoptosis by fish oil diet. We showed that in fish oil fed tumors the anti-apoptotic protein Bcl_{XL} expression was significantly reduced while the expression of the pro-apoptotic protein cytochrome-c increased indicating an activation of apoptosis in this group of tumors. We examined the status of another anti-apoptotic protein Bcl-2. Expression of Bcl-2 was significantly reduced in the tumor samples derived from fish oil-fed mice. Many members of the caspase family, a class of cysteine proteases, are involved in apoptosis. The executor caspase, caspase-3, cleaves various cellular targets and thus facilitates cell death. Using both western blotting and an enzymatic assay for detecting activated caspase 3 in tumor lysates we found an increase in caspase 3 activation in tumors of mice fed fish oil diet, confirming increased apoptosis in these tumor samples. To confirm the fish-oil induced tumor apoptosis, we examined the effect of two polyunsaturated fatty acids DHA and EPA on the apoptosis of MDA MB-231 breast cancer cells. Both DHA and EPA induced death of the breast tumor cells as determined by the Annexin V binding to the damaged membrane of the apoptotic cells.

In addition we tested fish oil diet, rich in ω-3 polyunsaturated fatty acids (PUFAs) such as DHA and EPA, for its effectiveness in preventing bone metastasis of breast cancer cells. We also reported that recombinant BMP-2 decreased breast cancer cell proliferation in culture (1,2) and our preliminary data suggest that BMP-2 can inhibit tumor growth in a breast cancer xenograft animal model. Therefore we tested whether BMP-2 can inhibit bone metastasis of the breast cancer cells using an animal model of bone metastasis. Our preliminary results suggest that BMP-2 expression in breast cancer cells decreased bone metastasis of these cells. Our aim for this proposal is to elucidate the molecular mechanism of fish oil-induced inhibition of breast cancer cell growth so that it could be used as a dietary supplemental therapy for the betterment of the life of the breast cancer patients.

Body:

Task 1. To develop a new therapeutic approach using dietary fish oil for treating breast cancer cell growth and metastasis: (Months 1-12)

Determine the effect of fish oil on **bone metastasis** of breast cancer cells: We have injected the breast cancer cells into the left cardiac ventricle of nude mice and have monitored the metastatic progression of the cells in bone by radiography measurement of the lesions. Our preliminary assessment suggests that fish oil diet has reduced osteolysis in nude mice. We are now continuing to find the statistical significance of our observation by repeating the experiment using a larger animal group.

Task 2: Study the underlying mechanism of fish oil-induced growth inhibition of breast cancer cells in animal and tissue culture models: (Months 1-12)

In order to study the mechanism of activation of programmed cell death in tumors of fish oil fed animals we examined expression patterns of Bcl_{XL} and Bcl-2 anti-apoptotic

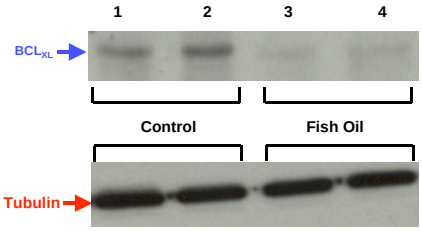

Figure 1. Effect of fish oil on Bcl_{XL} expression in tumor samples. Equal amounts of tumor lysates were immunoblotted with anti-Bcl_{XL} (top panel) and anti-tubulin (bottom panel) antibodies respectively. Expression of tubulin was used as loading control.

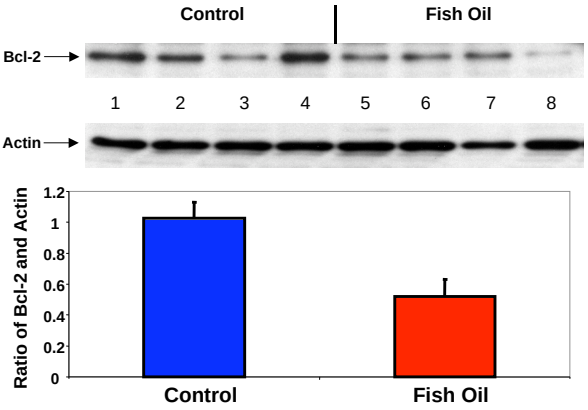

Figure 2. Effect of fish oil on expression of anti-apoptotic protein Bcl-2. Tumor lysates from mice fed fish oil diet and control diet were analyzed by western blotting using a Bcl-2 antibody. Middle panel shows anti-actin immunoblotting of the same samples to demonstrate loading of proteins. The expession of Bcl-2 was quantitated as ratio of intensity of Bcl-2 and actin (Bottom panel).

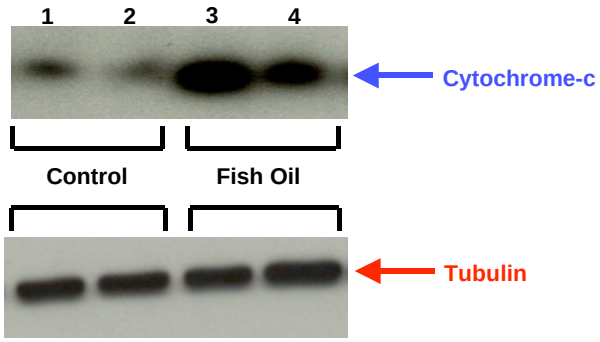

Figure 3. Effect of fish oil on cytochrome c expression in tumor samples. Equal amounts of tumor lysates were immunoblotted with anti-cytochrome c (top panel) and anti-tubulin (bottom panel) antibodies respectively. Expression of tubulin was used as loading control.

proteins. Expression of these proteins in the tumor lysates were examined by Western blot analysis. Their expression were reduced in the tumor lysates from fish oil fed animals as compared to that from the control animals (Figs. 1, 2). Actin/Tubulin immunoblotting was used as a loading control. The bottom barogram represents the quantitation of the Bcl-2 expression. We subsequently showed an increase in cytochrome c protein expression in tumors from animals fed fish oil diet. Release of cytochrome c from mitochondria activates the caspase cascade and thus initiates apoptotic death of the cells. Therefore, we studied activation of caspase 3, the executor caspase, in tumors from fish oil fed animals. Caspase 3 is activated by cleaving from its precursor procaspase 3 (33 kD) by the initiator caspase 9. Thus production of a small molecular weight (17 kD) protein from procaspase 3 represents the activated caspase 3. Western blot analysis of tumor lysates with a caspase 3 antibody that recognizes both forms showed increased production of 17 kD activated caspase 3 in the fish oil fed animals (Fig. 4), indicating induction of apoptosis in fish oil fed tumors leading to less tumor burden. Actin immunoblotting was used for loading control.

To investigate the mechanism, we examined the effect of the fatty acids DHA and EPA on MDA MB-231 cells in culture. To detect cell death, plasma membrane expression of phosphatidylserine as a measure of changes in apoptotic cells was analyzed. FITC-tagged Annexin V binding to MDA MB-231 cells after incubation with DHA or EPA. Additionally propidium iodide was used to distinguish between viable and nonviable cells. Flow cytometric analysis showed significant apoptosis of breast tumor cells by both DHA and EPA (Fig. 5A and Fig. 5B). Quantitation of these data is shown in the right panel of Fig. 5A and 5B.

We examined the effect of these unsaturated fatty acids on transcription of Bcl_{XL} gene using a reporter plasmid in which the firefly luciferase gene is driven by Bcl_{XL} promoter region (Fig. 6). Thus luciferase activity represents the transcription of Bcl_{XL} gene. The reporter was transiently transfected into MDA MB-231 cells followed by incubation of the cells with DHA and EPA. Luciferase activity in the cell lysates was determined. Both DHA and EPA significantly prevented ($p = 0.0075$) transcription of Bcl_{XL} in these breast tumor cells (Fig.7).

Similarly, transient transfection assay of Bcl-2 promoter driven luciferase into MDA MB-231 cells showed reduced Bcl-2 transcription by both DHA and EPA (Fig. 6 A and Fig. 6B).

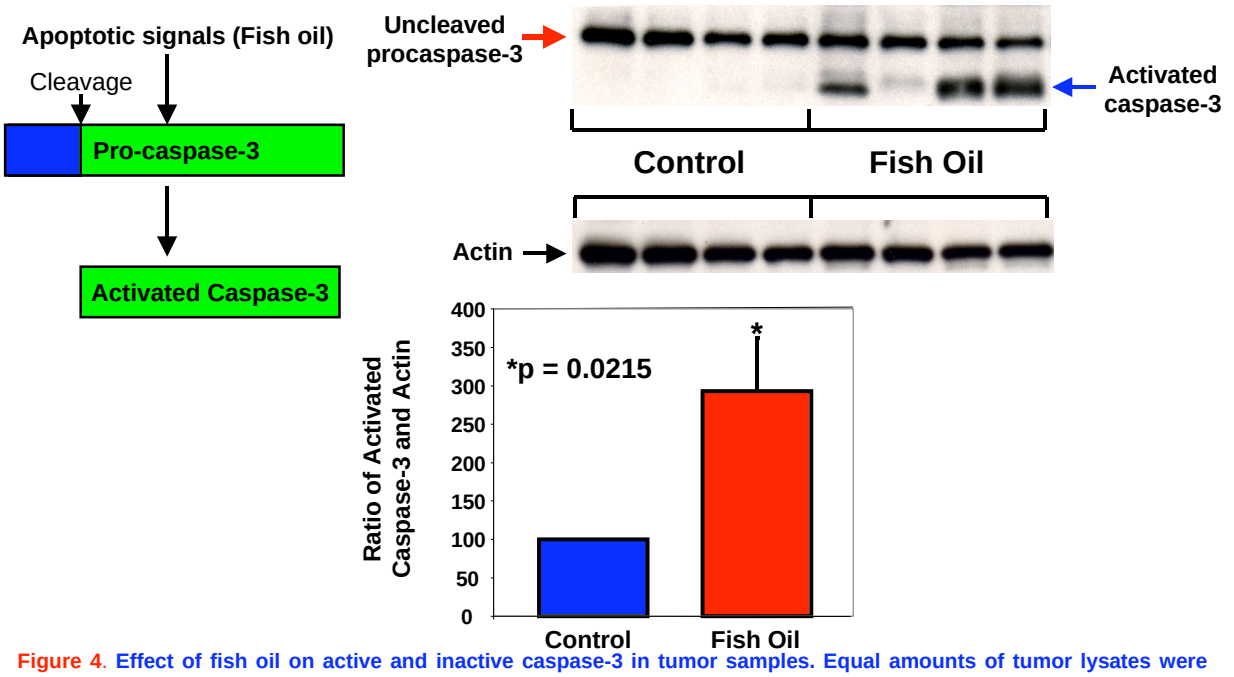

Figure 4. Effect of fish oil on active and inactive caspase-3 in tumor samples. Equal amounts of tumor lysates were immunoblotted with caspase-3 (top panel) and anti-actin (bottom panel) antibodies respectively. Expression of actin was used as loading control. Bottom graph shows the significant increase in cleaved active capsase-3 in the fish oil-fed samples.

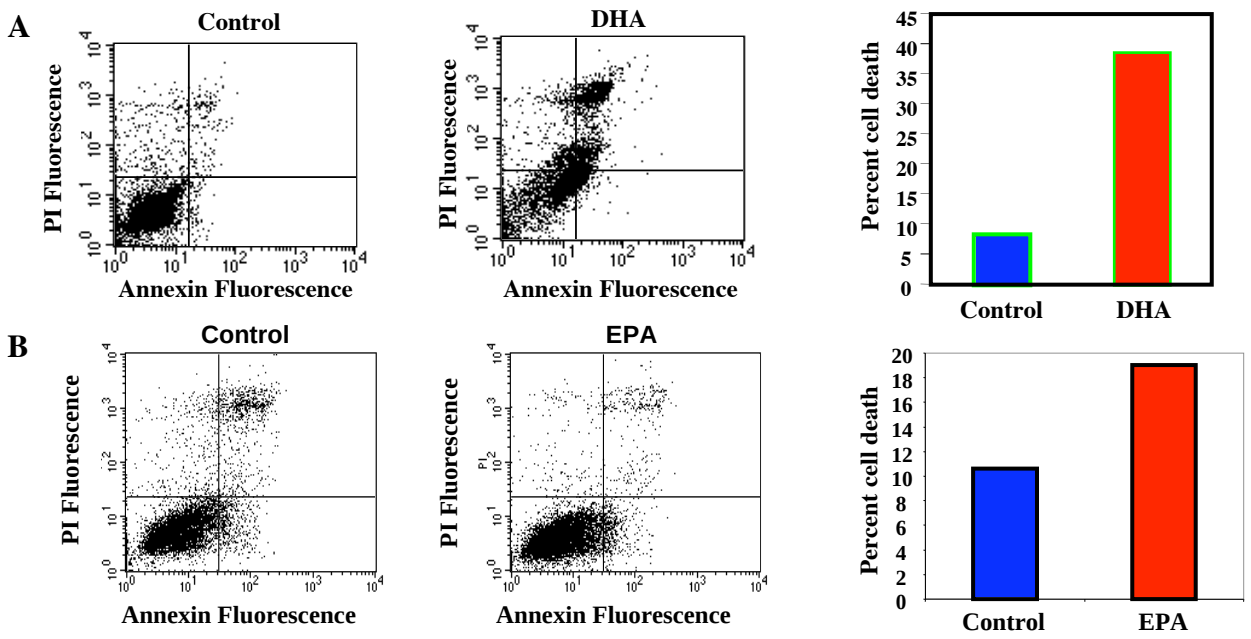

Figure 5. Effect of DHA and EPA on breast tumor cells. MDA MB-231 breast tumor cells were incubated with DHA (panel A) or EPA (panel B) for 24 hour. The cells were trypsinized and washed with saline and stained with fluoresceinisothiocyanate (FITC)-tagged Annexin V and propidium iodide (PI) using a commercially available kit as described above. Cells were analyzed in Flow Cytometer. The percentage of cell death in upper chambers and lower right (LR) chamber were plotted and shown in the right as bar graph.

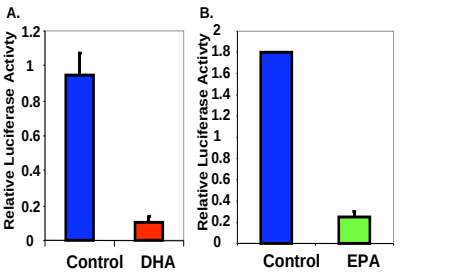

Figure 6. Effect of DHA and EPA on Bcl-2 transcription. Bcl-2 promoter driven luciferase plasmid was transfected into MDA-MB-231 breast cancer cells. Transiently transfected cells were treated with (A) DHA and (B) EPA. Luciferase activity was measured using an assay kit from Promega Inc.

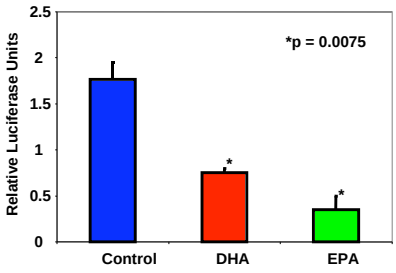

Figure 7. Effect of DHA and EPA on Bcl_{XL} transcription. Bcl_{XL} promoter driven luciferase plasmid was transfected into MDA-MB-231 breast cancer cells. Transiently transfected cells were treated with DHA and EPA. Luciferase activity was measured using an assay kit from Promega Inc.

Task 3: Test therapeutic efficacy of BMP-2 to inhibit breast cancer cell growth and bone metastasis in a mouse model using adenovirus vector**:** (Months 1-12)

To assess the effect of BMP-2 *in vivo* in tumor cell growth, we injected MDA MB 231 cells infected with either Ad-BMP-2 (an adenovirus vector expressing BMP-2) or a control Ad-β-Gal into nude mouse mammary fat pad. Initial pilot experiment showed less bone metastasis of the breast cancer cells marked by decreased area of osteolysis in this group of animals.
We are currently planning for repeating this experiment with a larger animal pool to find the statistical significance of our data.

Key Research Accomplishments throughout the total project duration:

- We demonstrated that fish oil diet significantly reduced tumor size in a xenograft model of MDA MB 231 cells.
- BMP-2 expression can also significantly reduce tumor size in the MDA MB 231 breast cancer xenograft model.
- As a molecular mechanism of this reduction we show that

A) fish oil diet can induce the PTEN tumor suppressor protein expression in xenograft tumors.
B) Fish oil diet can reduce the PI 3 kinase signaling pathway thus blocking a major cell survival pathway in this xenograft model.
C) Increased expression of cytochrome c in the tumors of fish oil-fed animal resulted in increased apoptosis of cancer cells leading to less tumor burden.
D) Less Bcl_{XL} expression may be the reason, why the tumors in fish oil group were smaller than that in the control group.
E) The high level of DHA and EPA present in the fish oil may be the reason for reduced breast tumor growth in fish oil fed animals.

Reportable Outcomes:

Part of this work has been presented as an Abstract in the Era of Hope Conference (June, 2005).

Molecular mechanism of beneficial effect of fish oil in breast tumor growth

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Conclusions:

We provide data to prove the role of fish oil diet in inhibiting breast tumor growth in mice and the underlying molecular mechanism. In previous report, we demonstrated that fish oil diet is activating expression of a tumor suppressor protein, PTEN in breast tumors fed 10% fish oil supplements. This finding is supported by the subsequent loss of activity of PI 3 kinase, a key member of cell survival pathway. Additionally we showed that BMP-2 expression in breast cancer cells also significantly inhibits breast tumor growth in a xenograft model. We have preliminary data to suggest that the fish oil diet and BMP-2 may prevent bone metastasis of breast cancer cells. This year we have provided data to show that fish oil diet can induce apoptotic signaling in breast cancer cells. Together our approach provides exciting data that can be extended to use fish oil diet as a potential therapy for preventing breast cancer cell growth at the primary as well as the secondary bone sites.

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